

Serum Albumin Is Inversely Associated With Portal Vein Thrombosis in Cirrhosis

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We analyzed whether serum albumin is independently associated with portal vein thrombosis (PVT) in liver cirrhosis (LC) and if a biologic plausibility exists. This study was divided into three parts. In part 1 (retrospective analysis), 753 consecutive patients with LC with ultrasound-detected PVT were retrospectively analyzed. In part 2, 112 patients with LC and 56 matched controls were entered in the cross-sectional study. In part 3, 5 patients with cirrhosis were entered in the *in vivo* study and 4 healthy subjects (HSs) were entered in the *in vitro* study to explore if albumin may affect platelet activation by modulating oxidative stress. In the 753 patients with LC, the prevalence of PVT was 16.7%; logistic analysis showed that only age (odds ratio [OR], 1.024; $P = 0.012$) and serum albumin (OR, -0.422 ; $P = 0.0001$) significantly predicted patients with PVT. Analyzing the 112 patients with LC and controls, soluble clusters of differentiation (CD)40-ligand ($P = 0.0238$), soluble Nox2-derived peptide (sNox2-dp; $P < 0.0001$), and urinary excretion of isoprostanes ($P = 0.0078$) were higher in patients with LC. In LC, albumin was correlated with sCD40L (Spearman's rank correlation coefficient [r_s], -0.33 ; $P < 0.001$), sNox2-dp (r_s , -0.57 ; $P < 0.0001$), and urinary excretion of isoprostanes (r_s , -0.48 ; $P < 0.0001$) levels. The *in vivo* study showed a progressive decrease in platelet aggregation, sNox2-dp, and urinary 8-iso prostaglandin F 2α -III formation 2 hours and 3 days after albumin infusion. Finally, platelet aggregation, sNox2-dp, and isoprostane formation significantly decreased in platelets from HSs incubated with scalar concentrations of albumin. **Conclusion:** Low serum albumin in LC is associated with PVT, suggesting that albumin could be a modulator of the hemostatic system through interference with mechanisms regulating platelet activation. (*Hepatology Communications* 2019;3:504-512).

There is overwhelming evidence that cirrhosis may be complicated not only by bleeding episodes but also by venous thrombosis, which occurs mainly in the portal vein but may also be detected in the systemic circulation.⁽¹⁾ The prevalence of portal vein thrombosis (PVT) is approximately 15% in cirrhosis, whereas its incidence is roughly 2% per year in patients without a history of venous thrombosis.⁽²⁻⁶⁾ PVT occurs more frequently

in patients with cirrhosis who are 67 years of age in average or in those with a history of venous thrombosis or hepatocellular carcinoma (HCC).⁽⁵⁾ Liver decompensation is another important factor associated with PVT, as demonstrated by a more frequent association between PVT and Child-Pugh classes B and C; however, the underlying mechanism is still undefined.⁽⁵⁻⁷⁾ Venous stasis, which is a key component of the Virchow triad, likely plays a role in

Abbreviations: 8-iso-PGF 2α -III, 8-iso prostaglandin F 2α -III; CD, clusters of differentiation; HCC, hepatocellular carcinoma; HS, healthy subject; LC, liver cirrhosis; OR, odds ratio; PRO-LIVER, Portal Vein Thrombosis Relevance on Liver Cirrhosis; PRP, platelet-rich plasma; PT, prothrombin time; PVT, portal vein thrombosis; ROS, reactive oxidant species; sNox2-dp, soluble Nox2-derived peptide.

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patients with advanced disease, but the contribution of other mechanisms implicated, for instance, in clotting or platelet activation, is poorly understood. We have previously shown that serum albumin, which is a component of the Child-Pugh score, is lower in patients with cirrhosis with PVT compared to those without, but it is still unclear whether this finding is a mere epiphenomenon of the underlying liver disease, thereby unrelated to PVT development, or conversely has a pathophysiologic value in the context of clotting/platelet activation and eventually thrombosis.⁽⁵⁾ Because previous studies demonstrated an association between serum albumin and thrombosis in the setting of cardiovascular disease,^(8,9) the aim of the present study was to investigate if serum albumin was associated with PVT independently from Child-Pugh score and if a biologic plausibility between serum albumin and thrombosis exists. As to the latter, we performed *in vivo* and *in vitro* studies to explore if albumin affects platelet activation and if so, what the underlying mechanism is, focusing in particular on its potential capability of modulating intracellular oxidative stress.

Patients and Methods

We divided this study into three parts. Part 1 was a retrospective analysis of the association between PVT and Child-Pugh score components (ascites, encephalopathy, bilirubin, prothrombin time–international normalized ratio [PT-INR], albumin), age, and sex. Part 2 was a cross-sectional study to analyze the correlation between serum albumin, one of the laboratory

variables included in the Child-Pugh score, and platelet activation and oxidative stress indexes. Part 3 was an *in vivo* and *in vitro* investigation studying the possible influence of albumin on platelet activation by modulation of oxidative stress.

PATIENTS

In part 1, 753 (68% men; 64 ± 12 years) patients were enrolled in the Portal Vein Thrombosis Relevance on Liver Cirrhosis: Italian Venous Thrombotic Events Registry (PRO-LIVER; ClinicalTrials.gov identifier, NCT01470547).⁽⁵⁾ The study was an Italian multicenter study with the primary aim of estimating the prevalence of PVT in a cohort of patients with liver cirrhosis (LC). Patients with LC of any etiology and grade who were referred to any of the 43 participating centers were consecutively enrolled in the study. At baseline, each patient underwent a Doppler ultrasound examination of the portal vein and its branches to assess the presence of PVT. At enrollment, data on medical history, comorbidities, and severity of cirrhosis were registered (measured by Child-Pugh and Model for End-Stage Liver Disease scores) as well as several laboratory data (including serum creatinine, serum albumin, total bilirubin, and PT). PVT was first suspected when solid endoluminal material was detected in the main trunk of the portal vein and/or its branches, and it was then confirmed by demonstrating a filling defect on Doppler examination. PVT was classified as complete/incomplete according to the absence/presence of any residual blood flow. According to these criteria, PVT was detected in 17% ($n = 126$) of patients.

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Clinical and laboratory characteristics of patients according to the presence or absence of PVT have been depicted.⁽⁵⁾ Briefly, patients with LC with PVT were older and showed a more advanced and decompensated disease with higher prevalence of Child-Pugh B and C classes (63% versus 44%) than patients without PVT. Patients with PVT more often had a history of previous recanalized PVT (20% versus 4%) and higher HCC prevalence (35% versus 17%).

In part 2, a second cohort of patients with LC was enrolled to analyze the interplay between serum albumin levels and markers of platelet activation/oxidative stress. For this purpose, 112 patients (65 of A, 31 of B, and 16 of C Child-Pugh classes) without history of previous PVT, concomitant HCC, and free from PVT at enrollment were included in the study. As a control group, 56 patients without LC, matched for the presence of concomitant atherosclerotic risks and not taking any drug interfering with the clotting system, were selected (Table 1). Blood and urine samples of every study subject were stored at -80°C until use. The study was conducted in accordance with the European Union Note for Guidance of Good Clinical Practice and the Declaration of Helsinki. Informed consent was obtained for each participant prior to inclusion in the study.

TABLE 1. CLINICAL AND LABORATORY CHARACTERISTICS OF PATIENTS WITH CIRRHOSIS WITHOUT PORTAL VEIN THROMBOSIS AND MATCHED CONTROLS (PART 2)

	Controls (n = 56)	Cirrhosis (n = 112)	P
Age, years	64 \pm 13	66 \pm 8	0.3559*
Sex male, n (%)	33 (59)	67 (60)	0.9115*
Comorbidities:			
CVD, n (%)	17 (30)	27 (24)	0.3850*
Diabetes, n (%)	22 (39)	39 (35)	0.3217*
Serum albumin (g/dL)	3.74 \pm 0.42	3.40 \pm 0.58	0.0002*
sCD40L (ng/mL)	8.0 [5.0-10.0]	9.5 [5.0-16]	0.0238 [†]
sNox2-dp (pg/mL)	11.9 [9.0-13.6]	24.0 [15.0-36.5]	<0.0001 [†]
Urinary 8-iso-PGF2 α -III (pg/mg creatinine)	145.0 [125.0-155.5]	260.0 [122.5-438.0]	<0.001 [†]

Data are expressed as median [IQR] or mean \pm SD.

*t test; [†]Mann-Whitney U Test; #chi-square test.

Abbreviation: CVD, cardiovascular disease.

In part 3, we enrolled 5 patients with cirrhosis (4 male patients and 1 female patient; aged 54-78 years). During hospitalization, they received infusion of human albumin (40 g/day) for 3 days; thereafter, blood samples were taken 4 days after albumin discontinuation.

Blood was collected in vacutainer tubes with and without anticoagulant between 8:00 and 9:00 AM at four time points: baseline, 2 hours later, at 3 days, and at 7 days. Samples were centrifuged for 15 minutes at 180g for analysis of platelet aggregation or 10 minutes at 300g for analysis of soluble Nox2-derived peptide (sNox2-dp), as reported below. Urinary samples were taken at the same time for the analysis of 8-iso prostaglandin F2 α -III (8-iso-PGF2 α -III).

IN VITRO STUDY

Platelet Preparation and Aggregation

To obtain platelet-rich plasma (PRP), citrated blood samples taken from healthy subjects (HSs) (n = 4; male patients 2, female patients 2; aged 39.7 \pm 7.6 years) were centrifuged for 15 minutes at 180g. To avoid leukocyte contamination, only the top 75% of the PRP was collected according to Pignatelli et al.⁽¹⁰⁾ Platelet-poor plasma was prepared by centrifuging the remaining sample at 300g for 10 minutes at room temperature. PRP was stimulated with collagen (2 $\mu\text{g/mL}$), and light transmission was recorded over 10 minutes in a two dual-channel module ChronoLog Model 700 light transmission aggregometer.

To obtain washed platelets for *in vitro* study, PRP was treated with acid citrate dextrose (10/1 volume [vol]/vol), centrifuged at 300g for 10 minutes (twice) in the presence of prostaglandin E1 (1 μM), and finally suspended (2×10^8 platelets/mL) in albumin-free Tyrode's buffer containing 137 mmol/L NaCl, 0.3 mmol/L Na₂HPO₄, 2 mmol/L KCl, 12 NaHCO₃, 5 mmol/L N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, 5 mmol/L glucose pH 7.3, and 1 mM CaCl₂.

Washed platelets were pre-incubated (15 minutes at 37°C) with albumin at 2, 2.5, 3, 3.5, and 4 g/dL, activated with collagen (2 $\mu\text{g/mL}$), and light transmission recorded. Experiments were performed at 37°C in the presence of 50 $\mu\text{g/mL}$ fibrinogen and under stirring conditions.

After stimulation with agonist, samples were centrifuged for 3 minutes at 3,000g. Supernatants were

stored at -80°C for analysis of sNox2-dp levels and 8-Iso-PGF2 α -III formation. The intra-assay and interassay coefficients of variation for aggregation test were 5.7% and 6.8%, respectively.

Integrin $\alpha\text{IIb}\beta 3$ Activation

Platelets washed in albumin-free Tyrode's buffer were diluted to a concentration of 3×10^7 platelets/mL in Tyrode's buffer containing 0, 2.5, 3, 3.5, and 4 mg/dL of bovine serum albumin (fraction V; Sigma-Aldrich). After 15 minutes of rest, platelets were activated with 10 ng/mL convulxin (Kenneth Clemetson, Theodor Kocher Institute, University of Berne, Switzerland) in the presence of 1 mM CaCl_2 and 5 $\mu\text{g/mL}$ pituitary adenylate cyclase-activating polypeptide type I receptor-fluorescein isothiocyanate (BD Biosciences), an antibody directed toward the activated form of human $\alpha\text{IIb}\beta 3$. Following 10 minutes of incubation, samples were diluted with 1 mL of phosphate-buffered saline and analyzed immediately with a BD Accuri C6 Plus flow cytometer (BD Biosciences). Data are shown as the percentage of maximal activation (mean \pm SD).

Serum and Platelet sNox2-dp

Serum and platelet Nox2 activity were measured as sNox2-dp with an enzyme-linked immunosorbent assay (ELISA) method as reported.⁽¹¹⁾ Briefly, reference standards of known concentrations of sNox2-dp and samples (1 mg of protein) were coated into ELISA 96-well plates overnight at 4°C . Anti-sNox2-dp-horseradish peroxidase (HRP) monoclonal antibody was added to each well, and immobilized antibody enzyme conjugates were quantified by monitoring HRP activities in the presence of the substrate 3,3',5,5'-tetramethylbenzidine. Enzyme activity was measured spectrophotometrically by the increased absorbency at 450 nm after acidification of formed products (2 M sulphuric acid). Values were expressed as pg/mL; intra-assay and interassay coefficients of variation were $<10\%$.

Urinary and Platelet 8-Iso-PGF2 α Assays

Urinary and platelet isoprostane (8-iso-PGF2 α -III) were measured by the enzyme immunoassay method

(DRG International, Springfield Township, NJ) and expressed as pg/mg creatinine or pmol/L. Intra-assay and interassay coefficients of variation were 5.8% and 5.0%, respectively.

Plasma sCD40L

Soluble clusters of differentiation (sCD)40L was measured in citrated blood samples centrifuged 10 minutes at 300g. sCD40L levels were evaluated by a commercial immunoassay (DRG International), and values were expressed as ng/mL; intra-assay and inter-assay coefficients of variation were $<10\%$.

STATISTICAL ANALYSIS

All continuous variables were tested for normality with the Shapiro-Wilk test. Variables with normal distribution were expressed as mean \pm SD and tested for differences with the Student t test with, if necessary, Bonferroni's correction. Non-normal variables were expressed as median and interquartile range, and differences were tested with the Mann-Whitney U test. Multiple linear regression and logistic regression analyses were also performed. Stochastic level of entry into the model was set at $P = 0.10$, and interaction terms were explored for all the variables in the final model. Only $P < 0.05$ was regarded as statistically significant. All tests were two tailed, and analyses were performed using computer software packages (SPSS, version 22.0; IBM, Armonk, NY).

Results

PART 1

In accordance with our previous report, 126 patients with PVT at baseline had lower albumin serum levels (3.1 ± 0.6 versus 3.4 ± 0.6 g/dL; $P < 0.0001$) than 627 patients without PVT.⁽⁵⁾ Comparing patients with PVT with Child-Pugh A ($n = 45$ out of 397; 11%) versus those in classes B and C ($n = 81$ out of 356; 23%), PVT was consistently associated with lower levels of serum albumin (Fig. 1A,B). We used multiple regression to analyze the association between Child-Pugh score components (ascites, encephalopathy, bilirubin, PT-INR, albumin), age, sex, and PVT. The final model (F [regression mean square] = 4.5;

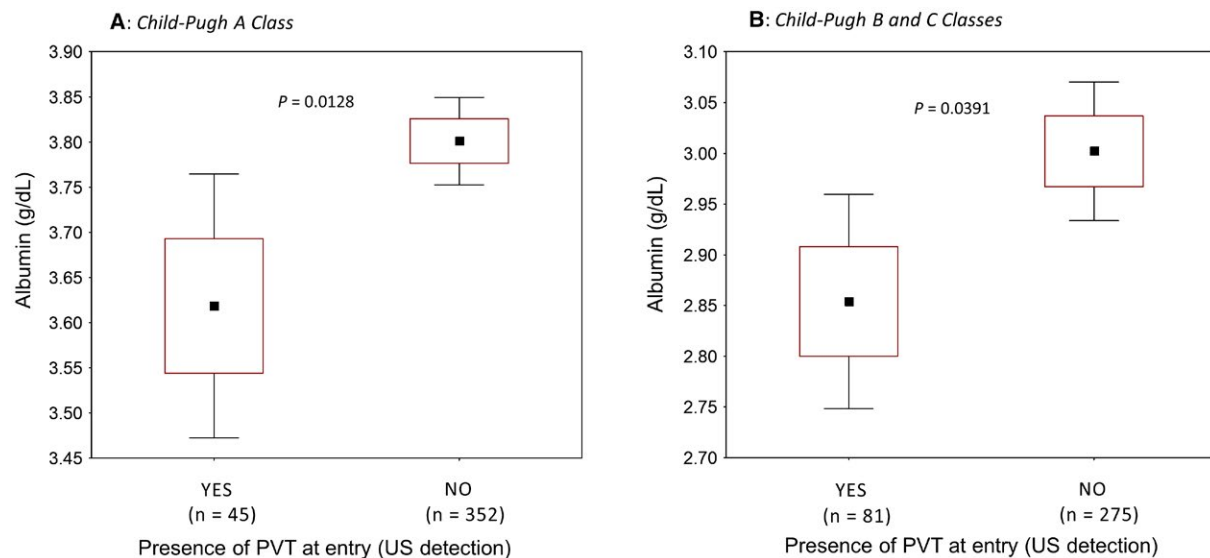


FIG. 1. Albumin serum levels according to presence of venous portal thrombosis in patients with liver cirrhosis ($n = 753$). (A) Patients with liver cirrhosis in Child-Pugh A. (B) Patients with liver cirrhosis in Child-Pugh B and C classes. Abbreviation: US, ultrasound.

$P < 0.0006$) showed that only age (beta coefficient, 0.10; SEM, 0.04; $P = 0.014$) and serum albumin (beta coefficient, -0.14 ; SEM, 0.05; $P = 0.0051$) were independently associated with PVT. By excluding the 152 patients with HCC at baseline, the multivariate analysis confirmed that only serum albumin was significantly inversely correlated with PVT (beta coefficient, -0.16 ; SEM, 0.06; $P = 0.0042$).

To corroborate the data regarding the relation between PVT at entry and Child-Pugh score components, a logistic regression analysis was also performed; the final model (stepwise forward method) confirmed that only age (odds ratio [OR], 1.024; 95% confidence interval [CI], 1.005-1.043; $P = 0.012$) and serum albumin (OR, 0.422; 95% CI, 0.293-0.608; $P = 0.0001$) significantly predicted patients with PVT.

PART 2

Analyzing the 112 patients with LC and matched control subjects, sCD40L plasma levels ($P = 0.0238$), sNox2-dp serum levels ($P < 0.0001$), as well as urinary excretion of isoprostanes ($P = 0.0078$) were significantly higher in LC than in control (Table 1). In patients with LC, serum albumin levels were significantly correlated with sCD40L ($r_s = -0.33$; $P < 0.001$), sNox2-dp serum levels ($r_s = -0.57$; $P < 0.0001$), and urinary

excretion of isoprostanes ($r_s = -0.48$; $P < 0.0001$) (Fig. 2A-C).

PART 3: IN VIVO STUDY

Serum albumin levels were 1.57 ± 0.51 g/dL at baseline (Fig. 3A) and progressively increased after 2 hours and 3 days (Fig. 3A); 4 days after albumin discontinuation, serum albumin tended to return to baseline values (Fig. 3A). Compared to baseline, platelet aggregation, serum levels of sNox2-dp, and urinary 8-iso-PGF 2α -III formation were significantly lowered (Fig. 3B-D); conversely, 4 days after albumin discontinuation, they all increased (Fig. 3B-D).

PART 3: IN VITRO STUDY

Platelets from healthy volunteers were incubated with scalar concentrations of albumin (2.5, 3, 3.5, and 4 g/dL). After stimulation, albumin-treated platelets showed a significant decrease of platelet aggregation (Fig. 4A) and α IIb β 3 activation (Fig. 4B) compared to controls; this effect was already evident at concentrations of 2.5 g/dL. Coincidentally, we found a significant reduction of platelet sNox2-dp levels and isoprostane formation (Fig. 4C,D).

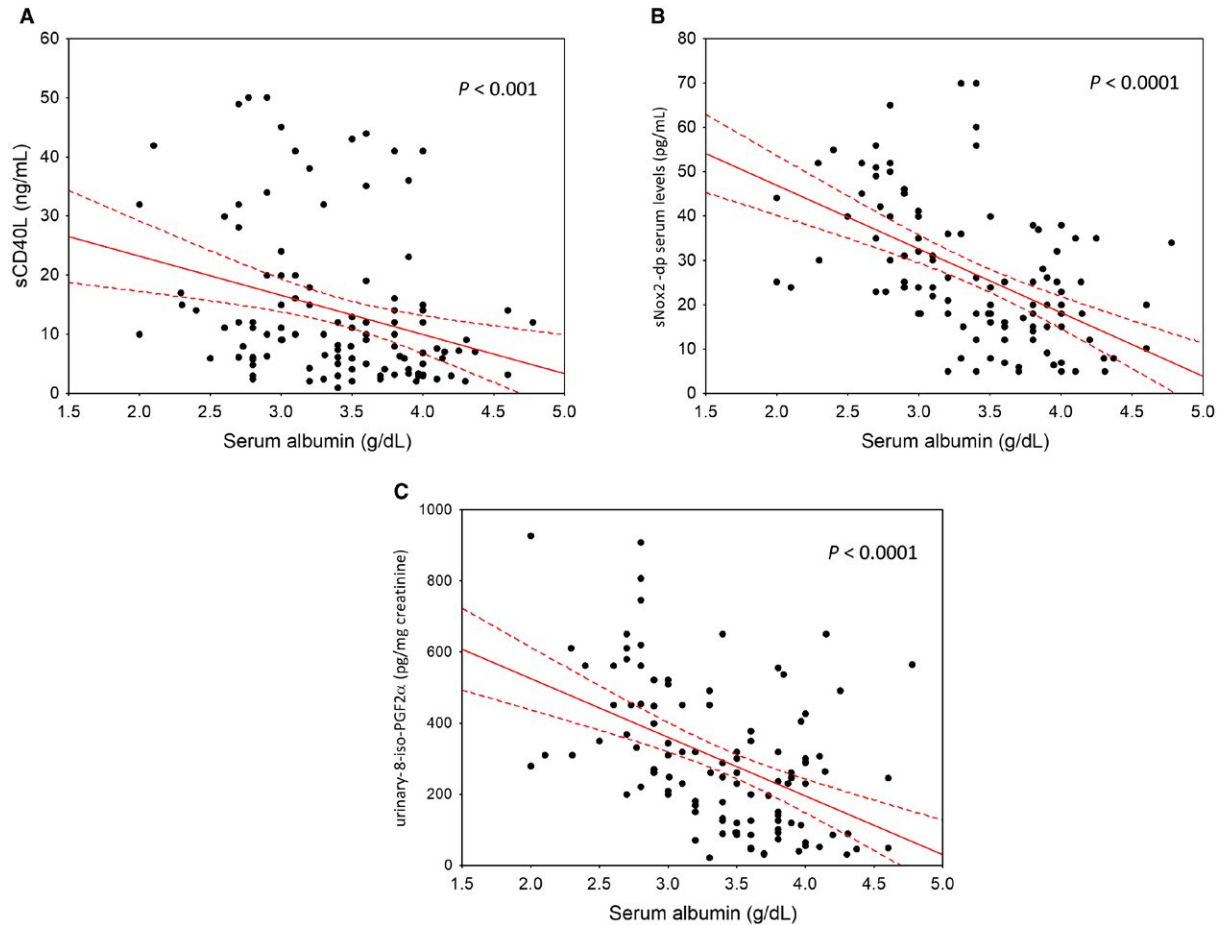


FIG. 2. Correlation of factors with serum albumin. Correlation between serum albumin levels and (A) sCD40L plasma levels, (B) sNox2-dp serum levels, and (C) urinary excretion of isoprostane in 112 patients with liver cirrhosis. Spearman's rank correlation coefficient (r_s) was calculated.

Discussion

This study provides evidence that serum albumin is inversely associated with PVT in patients with cirrhosis and suggests albumin as a modulator of the hemostatic system through interference with mechanisms regulating platelet activation. In particular, we demonstrate that albumin interferes with platelet activation by inhibiting Nox2-mediated oxidative stress.

Previous studies consistently showed that PVT is more often associated with advanced liver disease, but the underlying mechanism was unclear. In the present analysis of the PRO-LIVER study population, which investigated the predictors of PVT in patients with cirrhosis, we found that serum albumin was lower in patients presenting with PVT compared to those without PVT and was independently associated with PVT after adjustment for several factors, including

degree of liver failure as assessed by Child-Pugh score. In accordance with this, serum albumin was associated with PVT not only in patients of Child-Pugh classes B and C but also in those of class A, reinforcing the hypothesis that albumin *per se* may be a major determinant of PVT occurrence. Our data are consistent with previous reports showing an inverse relationship between serum albumin and arterial and venous thrombotic events.^(8,12)

To explore the biological plausibility of this finding, we performed *in vivo* and *in vitro* experiments to test the hypothesis that albumin interferes with mechanisms favoring the thrombotic process. In a first set of experiments, we investigated whether serum albumin is associated with markers of *in vivo* platelet activation. To explore this hypothesis, we analyzed a cohort of patients with cirrhosis with clinical characteristics similar to those of the PRO-LIVER study

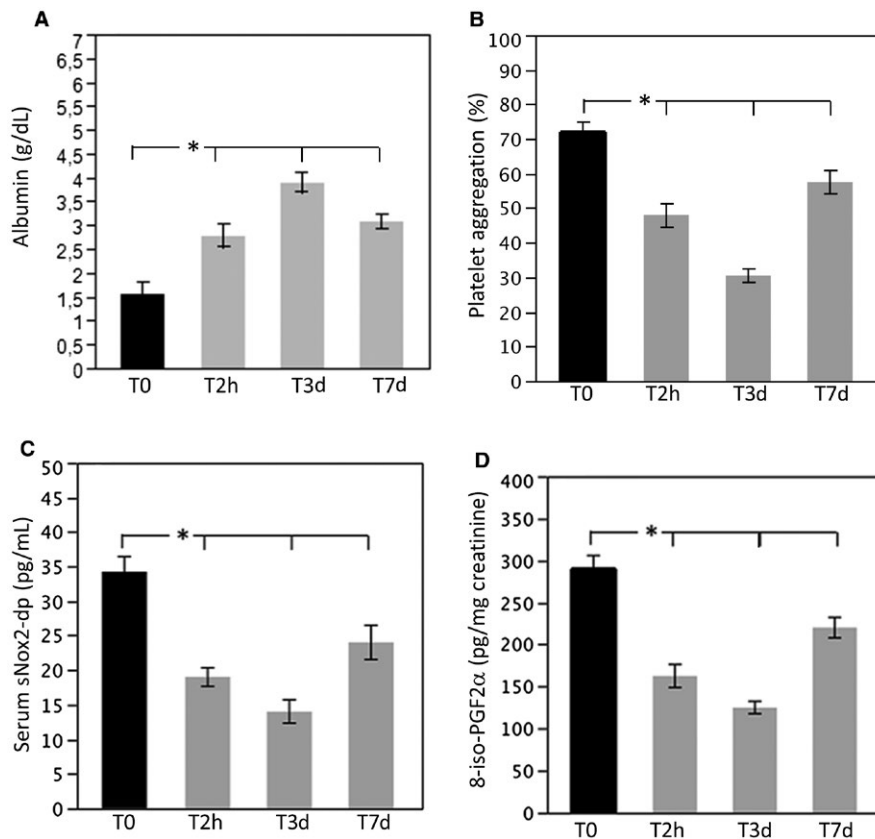


FIG. 3. Serum levels after albumin infusion. Serum levels of (A) albumin, (B) platelet aggregation, (C) sNox2-dp, and (D) urinary excretion of 8-iso-PGF2 α -III before time 0 (T0), 2 hours (T2h) later, 3 days (T3d) after albumin infusion, and 4 days after albumin discontinuation (T7d) in patients with liver cirrhosis (n = 4; * P < 0.05). *Bonferroni type adjustment was used for comparisons. Data represent mean \pm SEM.

and found that serum albumin inversely correlated with plasma sCD40L, which partially reflects *in vivo* platelet activation,⁽¹³⁾ as well as with markers of oxidative stress, such as sNox2-dp and urinary isoprostanes. The inverse association between serum albumin and oxidative stress is important for several reasons. First, albumin is known to exert an antioxidant effect by quenching reactive oxidant species (ROS) or binding and inactivating free metal, such as copper and iron, which otherwise would catalyze ROS formation.^(12,14) In the present study, we provide further insight into the albumin antioxidant property by showing an inverse association with the activation of Nox2, which is among the most important cellular producers of ROS and is implicated in platelet activation.^(15,16) To further substantiate the inverse association between serum albumin and Nox2 activation, we performed an *in vitro* study exposing platelets to scalar concentrations of albumin and demonstrated that albumin dose

dependently inhibits agonist-induced platelet activation, coincidentally with down-regulation of Nox2 activation and formation of isoprostanes, which are powerful platelet agonists⁽¹⁰⁾ and are produced on activation of Nox2.⁽¹⁰⁾ Inhibition of Nox2 activation is an important step in the mechanism of platelet activation because genetic deficiency of this enzyme is associated with impaired platelet activation^(16,17) and platelet-dependent thrombus formation.⁽¹⁰⁾ Such *in vitro* data were corroborated by a proof-of-concept study performed in 5 patients with cirrhosis who needed to be treated with albumin infusion. Thus, we found that, compared to baseline values, agonist-induced platelet aggregation progressively decreased for the 3 days of albumin infusion but later tended to return to baseline values at 4 days after albumin discontinuation. At the same time points, inhibition of platelet aggregation was associated with an antioxidant effect, as shown by decreasing sNox2 and isoprostane. Together, these

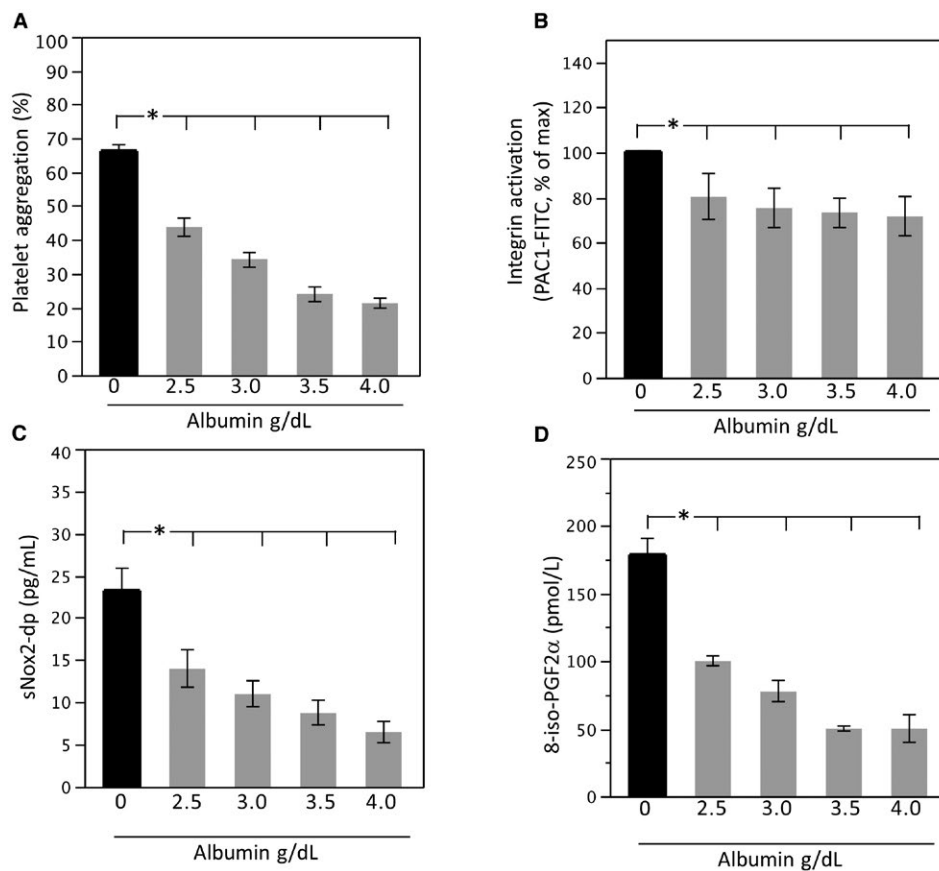


FIG. 4. Platelets from healthy subjects incubated with albumin. (A) Platelet aggregation, (B) Nox2 activation by sNox2-dp, and (C) isoprostane formation in platelets from HSs incubated with scalar concentrations (2.5, 3.0, 3.5, and 4.0 g/dL) of albumin ($n = 4$; $P < 0.05$). *Bonferroni type adjustment was used for comparisons. Data represent mean \pm SEM. Abbreviations: FITC, fluorescein isothiocyanate; PAC1, pituitary adenylate cyclase-activating polypeptide type I receptor.

findings suggest that albumin exerts an antiplatelet effect by inhibiting Nox2-derived ROS and eventually isoprostane formation.

The present study has implications and limitations. Due to the cross-sectional nature of the study, a cause-effect relationship between serum albumin and thrombosis cannot be firmly substantiated. However, the significant association between albumin and PVT provides a rationale to assess if albumin supplementation may be a therapeutic tool for PVT treatment. Finally, we have partial information to evaluate if changes of albumin over time predict PVT.

In conclusion, we provide the first evidence that serum albumin is inversely associated with PVT and a rationale for randomized interventional studies to investigate the beneficial effects of albumin to prevent PVT in cirrhosis.

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